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Urinary biomonitoring of subjects with different smoking habits. Part I: profiling mercapturic acids

Gianfranco Frigerio[1]*, Rosa Mercadante[1]*, Laura Campo[2], Elisa Polledri[1], Luca Boniardi[1], Luca Olgiati[2], Pasquale Missineo[2], Silvia Fustinoni[1,2]**

[1] Department of Clinical Sciences and Community Health, Università degli Studi di Milano, Milan, Italy

[2] Environmental and Industrial Toxicology Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy

* Both authors contributed equally to this work.

**Corresponding author.

Corresponding author

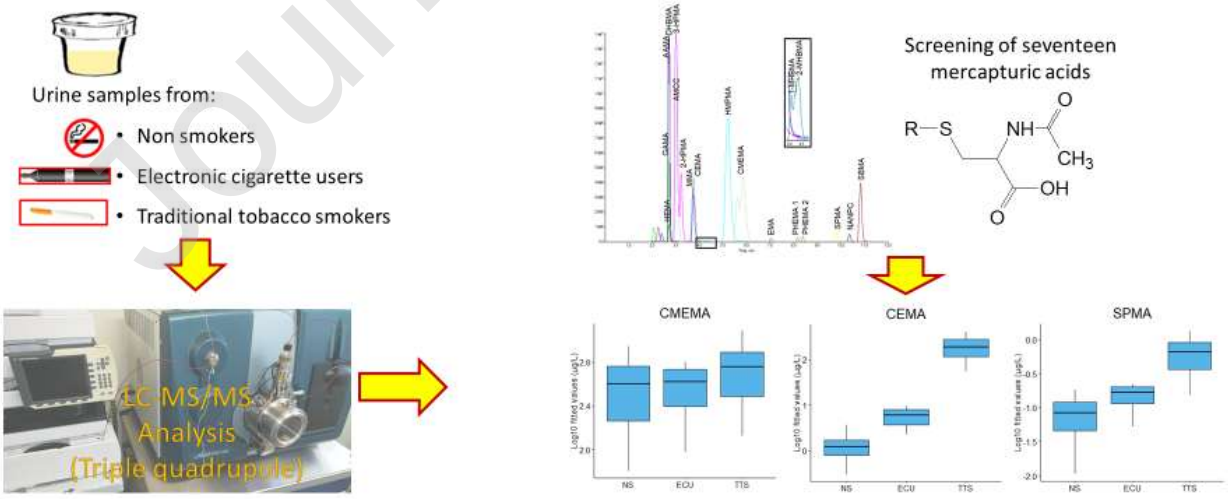
Silvia Fustinoni

Via S. Barnaba, 8 - 20122 Milan, Italy

e-mail: silvia.fustinoni@unimi.it

phone: +39 02 503 20158

Graphical abstract



Highlights

- 67 study subjects: 38 non-smokers, 7 e-cig users, and 22 tobacco smokers
- 17 urinary mercapturic acids, metabolites of carcinogenic/toxic chemicals measured
- Most mercapturic acids higher in tobacco smokers than in non-smokers
- Metabolites of acrylonitrile and acrolein higher in e-cig users than in non-smokers

Abstract

Background: While tobacco smoke contains thousands of chemicals, some of which are carcinogenic to humans, the content of electronic cigarette smoke is less known. This work aimed to assess and compare the exposure associated with different smoking habits by profiling urinary mercapturic acids as biomarkers of toxic compounds.

Methods: In this pilot study, sixty-seven healthy adults with different smoking habits were investigated: 38 non-smokers (NS), 7 electronic cigarette users (ECU), and 22 traditional tobacco smokers (TTS). Seventeen urinary mercapturic acids, metabolites of 1,3-butadiene (DHBMA, MHBMA), 4-chloronitrobenze (NANPC), acrolein (3-HPMA), acrylamide (AAMA, GAMA), acrylonitrile (CEMA), benzene (SPMA), crotonaldehyde (CMEMA, HMPMA), ethylating agents (EMA), methylating agents (MMA), ethylene oxide (HEMA), N,N-dimethylformamide (AMCC), propylene oxide (2-HPMA), styrene (PHEMA), and toluene (SBMA), were quantified, along with urinary nicotine and cotinine.

Results: Median urinary cotinine was 0.4, 1530 and 1772 µg/L in NS, ECU and TTS, respectively. Most mercapturic acids were 2 - 165 fold-higher in TTS compared to NS, with CEMA, MHBMA, 3-HPMA and SPMA showing the most relevant increases. Furthermore, some mercapturic acids were higher in ECU than NS; CEMA and 3-HPMA, in particular, showed significant increases and were 1.8 and 4.9 fold-higher, respectively.

Conclusions: This study confirms that tobacco smoking is a major source of carcinogenic chemicals such as benzene and 1,3-butadiene; electronic cigarette use is a minor source, mostly associated with exposure to chemicals with less carcinogenic potential such as acrylonitrile and acrolein.

Keywords

Urinary mercapturic acids; volatile organic compounds; smoking habit; tobacco smoking; electronic cigarette smoking; biomonitoring.

Abbreviation

2-HPMA, N-acetyl-S-(2-hydroxypropyl)cysteine; 3-HPMA, N-acetyl-S-(3-hydroxypropyl)cysteine; AAMA, N-acetyl-S-(carbamoylethyl)-L-cysteine; AMCC, N-acetyl-S-(N-methylcarbamoyl)-L-cysteine; ANOVA, analysis of variance; BMI, body mass index; CEMA, N-acetyl-S-(2-cyanoethyl)-L-cysteine; CMEMA, N-acetyl-S-(3-carboxy-2-propyl)-L-cysteine; DHBMA, N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine; ECU, electronic cigarette users; EMA, N-acetyl-S-ethyl-L-cysteine; GAMA, N-acetyl-S-(2-hydroxy-3-propionamide)-L-cysteine; GMR, Geometric Mean Ratio; HEMA, N-acetyl-S-(2-hydroxyethyl)-L-cysteine; HMPMA, N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LOQ, limit of quantitation; MHBMA, (R,S)-N-acetyl-S-[1-(hydroxymethyl)-2-propen-1-yl)-L-cysteine + (R,S)-N-acetyl-S-(2-hydroxy-3-buten-1-yl)-L-cysteine; MMA, N-acetyl-S-methyl-L-cysteine; NANPC, S-(4-nitrophenyl)mercapturic acid; NS, non-smokers; PHEMA, N-acetyl-S-(2-hydroxy-1-phenylethyl)-L-cysteine + N-acetyl-S-(2-hydroxy-2-phenylethyl)-L-cysteine; SBMA, N-acetyl-S-benzyl-L-cysteine; SPMA, N-acetyl-S-phenyl-L-cysteine; TTS, traditional tobacco smokers.

1. Introduction

Although tobacco smoking is carcinogenic to humans (Group 1 according to the International Agency for Research on Cancer, IARC) (IARC, 2004, 2012b), its prevalence is still considerable since smokers represent 22.5% of the global population (32.0% of males and 7.0% of females) (Gowing et al., 2015). In Italy, the prevalence is very similar to the global one since 22% of Italian people are smokers (28% of males and 16.5% of female) (Pacifici, 2019). Tobacco smoke is a mixture of chemicals containing more than 5000 compounds (Rodgman and Perfetti, 2013), among which over 70 have been classified as carcinogens (IARC, 2004, 2012b). The list of carcinogenic chemicals (IARC Group 1) includes 1,3-butadiene (IARC, 2012a), ethylene oxide (IARC, 2012a) and benzene (IARC, 2018a). Furthermore, probably carcinogenic chemicals (Group 2A) such as acrylamide (IARC, 1994) and styrene (IARC, 2018b) and possibly carcinogenic chemicals (Group 2B) such as propylene oxide (IARC, 1994) and acrylonitrile (IARC, 1999), are present in cigarette smoke (Table 1).

Besides traditional tobacco smoking, electronic cigarette is a relatively new product that is gaining increasing consideration. While traditional cigarettes use combustion to burn tobacco, electronic cigarettes use electricity to heat and aerosolize a liquid containing nicotine and flavourings. The prevalence of electronic cigarettes is increasing among both adolescents and adults (Breland et al., 2017), likely due to the low perceived risk associated with this smoking mode. Among Italian people, 1.7% uses electronic cigarette (Pacifici, 2019). While the presence of nicotine in the aerosol generated from the electronic cigarettes is considerable (Goniewicz et al., 2013), the amount of toxicants and carcinogens is lower if compared to traditional tobacco smoke (Goniewicz et al., 2014). Few evidences reported the presence of some toxic compounds in both vapour (Goniewicz et al., 2014; Laugesen, 2008; Schripp et al., 2013; Sleiman et al., 2016; Uchiyama et al., 2013) and liquid (Lim and Shin, 2017; Sleiman et al., 2016; Varlet et al., 2015) of electronic cigarettes (Table 1).

After being inhaled and absorbed, these compounds may undergo biotransformation to active electrophilic intermediates, which are able to react with DNA and, therefore, exert their genotoxic and carcinogenic potential (Parkinson and Ogilvie, 2010). In order to be deactivated, electrophilic compounds may undergone a conjugation with glutathione; then, after other enzymatic reactions, they can be finally excreted in urine as mercapturic acids (De Rooij et al., 1998). Biomonitoring through the quantitation of urinary levels of mercapturic acids is a suitable method for the assessment of environmental exposures to toxicants (Mathias and B'hymer, 2016). To achieve this goal, we recently developed a method to rapidly quantify several mercapturic acids simultaneously as biomarkers of different toxicants, including some carcinogens (Frigerio et al., 2019) (quantified analytes are reported in Table 1).

Although the internal dose of carcinogens assessed quantifying urinary mercapturic acid levels in traditional tobacco smokers has been extensively reported (Alwis et al., 2012; Chiang et al., 2015; Eckert et al., 2011; Li et al., 2015; Pluym et al., 2015; Schettgen et al., 2009; Schettgen et al., 2008; Zhang et al., 2014), only few evidences on electronic cigarette users are present, especially quantifying a wide range of mercapturic acids (Goniewicz et al., 2018; Keith et al., 2019). The aim of this pilot study was to assess exposure to different toxic chemicals, among which some carcinogens, in subjects with different smoking habits, namely non-smokers (NS), traditional tobacco smokers (TTS) and electronic cigarette users (ECU), using urinary mercapturic acids as biomarkers, and to compare the burden of toxicants associated with different smoking modes.

2. Materials and methods

2.1. Study subjects

The study involved 67 healthy workers, belonging to a plant recycling exhausted oil in Northern Italy. The plant, through a re-refining process, treats waste oils to produce regenerated bases which

are then used by other lubrication companies. The total treatment capacity is around 200,000 tonnes per year, producing mainly regenerated lube bases, but also diesel oil and a mixture for applications in bituminous membranes. Among enrolled subjects, both office workers (n = 9) and plant workers (n = 58) were present. Job tasks included plant management, exhaust oil receiving, remote and on-site plant control, plant maintenance, regenerated oil quality controls, regenerated oil storage and delivery.

For these workers a survey to assess the exposure to several chemicals, among which volatile organic compounds, was performed in June 2017. The survey protocol included the assessment of personal exposure during the work-shift and the biological monitoring, collecting a spot urine sample at the end of the shift. A questionnaire to collect personal and socio-demographic data (age, gender, nationality, height, body weight), smoking habit (mode and intensity, exposure to environmental tobacco smoking and daily length of exposure), lifestyle (commuting time and means of transport; car refuelling, use of solvents, dyes or paints in the spear time, and biomass burning in the previous 24 hours), diet (fried and barbecued food, coffee and alcohol intake in the previous 24 hours), and residential characteristics (rural, urban peripheral, or urban area, presence of industrial sites near residence, intensity of traffic at residence, presence of a car garage linked to house) was administered by the research team. Occupational activities were investigated through a detailed questionnaire including description of job tasks in the investigated work shift.

The research was conducted in the frame of the risk assessment activity, according to the Italian legislation D.Lgs. 81/08, for the protection of workers' health, under the supervision of the plant occupational health service. Each study subject read, understood and signed the informed consent form.

2.2. Urine collection and mercapturic acids analysis

Urine samples were stored on site at 4 °C and delivered to the laboratory within 72 h; once in the laboratory they were stored at -20 °C until analysis.

The urinary concentrations of N-acetyl-S-(2-hydroxypropyl)cysteine (2-HPMA), N-acetyl-S-(3-hydroxypropyl)cysteine (3-HPMA), N-acetyl-S-(carbamoylethyl)-L-cysteine (AAMA), N-acetyl-S-(N-methylcarbamoyl)-L-cysteine (AMCC), N-acetyl-S-(2-cyanoethyl)-L-cysteine (CEMA), N-acetyl-S-(3-carboxy-2-propyl)-L-cysteine (CMEMA), N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine (DHBMA), N-acetyl-S-ethyl-L-cysteine (EMA), N-acetyl-S-(2-hydroxy-3-propionamide)-L-cysteine (GAMA), N-acetyl-S-(2-hydroxyethyl)-L-cysteine (HEMA), N-acetyl-S-(3-hydroxypropyl-1-methyl)-L-cysteine (HMPMA), (R,S)-N-acetyl-S-[1-(hydroxymethyl)-2-propen-1-yl)-L-cysteine + (R,S)-N-acetyl-S-(2-hydroxy-3-buten-1-yl)-L-cysteine (MHBMA), N-acetyl-S-methyl-L-cysteine (MMA), S-(4-nitrophenyl)mercapturic acid (NANPC), N-acetyl-S-(2-hydroxy-1-phenylethyl)-L-cysteine + N-acetyl-S-(2-hydroxy-2-phenylethyl)-L-cysteine (PHEMA), N-acetyl-S-benzyl-L-cysteine (SBMA), and N-acetyl-S-phenyl-L-cysteine (SPMA) were measured (Table 1) with an LC-MS/MS method as previously described (Frigerio et al., 2019).

2.3. Urinary cotinine, nicotine and creatinine analysis

Cotinine and nicotine concentrations were quantified with an LC-MS/MS method (Fustinoni et al., 2013). The limit of quantitation was 0.1 µg/L for both cotinine and nicotine. Subjects with urinary cotinine >30 µg/L were classified as active consumer of nicotine products (either nicotine-based electronic cigarette users or traditional tobacco smokers) (Campo et al., 2016).

Creatinine in urine was measured using Jaffé reaction colorimetric method (Kroll et al., 1986).

2.4. Data processing and statistical analysis

The MultiQuant™ software (version 3.0.8664.0; Ab Sciex S.r.l, Milano, Italy) was used for chromatographic data integration. Statistical analysis was performed using both IBM SPSS® Statistics 25.0 for Windows (IBM, Armonk, New York, United States) and R (version 3.6.1, R Foundation, Vienna, Austria) (R-Core-Team, 2019) with the Rstudio interface (Version 1.2.1335, RStudio Inc., Boston, Massachusetts, United States) and the package “tidyverse” (Wickham, 2017).

A value corresponding to one-half of the LOQ was assigned to measurements below the analytical quantitation limit. Values were then adjusted for urinary creatinine and, for the descriptive analysis, median, 5^{th} and 95^{th} percentile values of the distribution were calculated. The variables were transformed into their decimal logarithms and a one-way analysis of variance (ANOVA) was applied to evaluate the difference among groups. A Bonferroni post-hoc test was also applied to evaluate differences between each group pairs.

For each mercapturic acid, two different multiple linear regression models were estimated. The first model (model A) was applied to evaluate the effect of cotinine on the urinary mercapturic acids while the second model (model B) aimed to evaluate the effect of the smoking mode on the concentration of urinary mercapturic acids. Different variables of the questionnaire were included in a preliminary analysis with simple linear models to test their contributions to the level of mercapturic acids but were excluded from the final models due to the lack of significant contribution. Then, multiple linear regression models were built as follows: the dependent variable in model A was the $\log_{10}$ urinary mercapturic acid (µg/L), while independent variables were: $\log_{10}$ cotinine (µg/L), $\log_{10}$ creatinine (g/L), age (years), body mass index (BMI) (kg/m^2), gender (male or female), and occupational exposure (office workers or plant workers). The dependent variable in model B was the $\log_{10}$ urinary mercapturic acid (µg/L), while independent variables were: smoking mode (NS or ECU or TTS), $\log_{10}$ creatinine (g/L), age (years), BMI (kg/m^2), gender (male or female), occupational exposure (office workers or plant workers).

The regression slopes were exponentiated to obtain the geometric mean ratio (GMR), representing the fold increase of geometric mean value of a mercapturic acid following a 10-fold increase in cotinine concentration for model A, and changing smoking mode from NS, taken as reference, to ECU and TTS, for model B.

3. Results

3.1. Subjects, cotinine, nicotine and creatinine levels

The main characteristics of study subjects are reported in Table 2. Of the 67 subjects, only 4 were females (2 plant workers and 2 office workers); the age of the subjects ranged from 27 to 62 years and BMI ranged from 19.0 to 37.0 kg/m^2. According to questionnaire, 39 subjects classified themselves as NS, 7 as ECU, and 21 as TTS; no dual smokers were present. All the products consumed by ECU were nicotine-based. No other details (i.e. type of electronic cigarette device, actual content of nicotine in liquid, and type of flavour) for further defining ECU habit were available. When the urinary cotinine cut-off (≥ 30 µg/L) was applied, one of the self-declared non-smokers showed a cotinine concentration much higher than the cut-off (1228 µg/L). After a further contact with the health personnel of the plant, the subject was reclassified in the TTS group. The number of cigarettes smoked per day in TTS ranged between 2 to 25.

Nicotine levels in NS were above LOQ in 19 out of 38 individuals (50%) with values not exceeding 2.34 µg/L, while cotinine levels in NS were above LOQ in 27 out of 38 individuals (71%) with values not exceeding 3.05 µg/L. Median nicotine levels in ECU and TTS were 2003 and 1456 µg/L, respectively; while cotinine medians were 1530 and 1772 µg/L. Both nicotine and cotinine levels were significantly higher in ECU vs NS ($P < 0.001$) and in TTS vs NS ($P < 0.001$) while they were similar in ECU vs TTS ($P = 1$).

Creatinine was similar in all groups: overall median was 1.5 g/L and values ranged between 0.3 and 3.1 g/L.

3.2. Mercapturic acid levels

The results from the analysis of urinary mercapturic acids, adjusted for creatinine, are reported in Table 3, along with the p-value of the Anova and Bonferroni post-hoc test. Results, not adjusted for creatinine, are reported in the Supplementary Table S1.

Mercapturic acid concentrations were above the LOQ for all samples for 2-HPMA, 3-HPMA, AAMA, AMCC, CMEMA, DHBMA, HMPMA, and SBMA; a few samples (1-22 %) had non-quantifiable levels of CEMA, GAMA, HEMA, MHBMA, MMA, PHEMA and SPMA; 42% of samples had non-quantifiable levels of EMA and almost all samples were non-quantifiable for NANPC (only 10% of samples above the LOQ). For this reason, NANPC was excluded from statistical analyses.

Mercapturic acid medians ranged between 0.03 (EMA in NS and ECU) and 1301 µg/g creatinine (3-HPMA in TTS). Anova test revealed significant differences among groups for all mercapturic acids, except for MMA and SBMA. After applying the Bonferroni post-hoc test, ECU had significantly higher levels than NS of CEMA ($p < 0.001$) and marginally significant for 3-HPMA ($p = 0.069$); TTS had significant higher levels than ECU of 2-HPMA, 3-HPMA, AAMA, CEMA, DHBMA, HMPMA, MHBMA, and SPMA; TTS had significantly higher levels than NS of all analytes, but MMA, and SBMA.

3.3. Multiple regression models

Results of the multiple linear regression analyses are shown in Table 4.

In the linear model A, the adjusted coefficient of determination (R^2) was significantly greater from zero for all analytes and ranged from 0.11 (EMA) to 0.72 (CEMA). The GMR for cotinine as determinant of urinary mercapturic acids was significantly greater than one for all compounds except for CMEMA, MMA, and SBMA; where significant, it ranged from 1.13 (DHBMA) to 2.92 (CEMA). The partial correlation coefficients (r) of cotinine were significantly greater than zero for all compounds except for MMA and SBMA; where significant, it ranged from 0.25 (CMEMA) to 0.85 (CEMA). The results regarding the other variables included in the linear model are here briefly described (data not reported in Table 4). As regards the occupational exposure, considering office workers as reference, the GMR associated with plant workers was increased only for SPMA (2.46 , 95%CI: 1.15 - 5.25, $p = 0.021$) and decreased for GAMA (0.55, 95%CI: 0.31 - 0.99, $p = 0.046$);

other mercapturic acids were not affected by occupational exposure. Considering gender and BMI: they were not significant determinants of any mercapturic acid; age was significantly associated with an increase of CMEMA, (p = 0.040), MHBMA (p = 0.031), MMA (p = 0.010), and SBMA (p = 0.020); creatinine was associated with an increase of all the considered mercapturic acids (p = 0.004, 0.007, and 0.030 for CEMA, EMA, and HEMA, respectively, and <0.001 for the other analytes) with partial correlation coefficient (r) ranging from 0.28 (HEMA) to 0.80 (DHBMA).

In the linear model B, the adjusted coefficient of determination (R^2) was significantly greater from zero for all analytes and ranged from 0.13 (EMA) to 0.92 (CEMA). The smoking habits influenced urinary levels of mercapturic acids with notable differences. Considering NS as the reference group, the GMR relative to ECU was significantly greater than one for 3-HPMA (1.78, p = 0.021) and for CEMA (4.85, p < 0.001); while the GMR relative to TTS was significantly greater than one for all analytes with the exception of MMA and SBMA; where significant, it ranged from 1.44 (CMEMA, p = 0.010) to 164.97 (CEMA, p < 0.001). Distributions of analytes, corrected for all variables included in this linear model, are visually reported as box-plot in Fig. 1-A and 1-B.

The results regarding the other variables included in the linear model are described below (data not reported in Table 4). As regards the occupational exposure, considering office workers as reference, the GMR associated with plant workers was increased for SPMA (2.83, 95%CI: 1.42 - 5.62, p = 0.004) and PHEMA (1.89, 95%CI: 0.95 - 3.77, p = 0.069) and decreased for GAMA (0.57, 95%CI: 0.31 - 1.03, p = 0.062), while the other mercapturic acids were not affected by occupational exposure. BMI was not a significant determinant of any mercapturic acid; gender was associated with significant higher levels of MHBMA in females (p = 0.031); age was significantly associated with an increase of CMEMA, (p = 0.026), MHBMA (p = 0.006), MMA (p = 0.010), and SBMA (p = 0.023); creatinine was associated with an increase of all the considered mercapturic acids (p = 0.005 and 0.025 for EMA and HEMA, respectively, and < 0.001 for all the other analytes) with partial correlation coefficient (r) ranging from 0.29 (HEMA) to 0.85 (DHBMA).

4. Discussion

We conducted a pilot biomonitoring study to assess the levels of several urinary mercapturic acids associated to carcinogenic and non-carcinogenic volatile organic chemicals in subjects with different smoking habits. As expected, the levels of most mercapturic acids were higher in TTS than in both NS and ECU; however, they were higher also in ECU compared to NS, showing an impact of this habit on the body burden of toxicants, mostly acrylonitrile and acrolein.

Among carcinogenic compounds (IARC Group 1), we found significantly higher concentrations of DHBMA and MHBMA (~2 and 20 fold) (metabolites of 1,3-butadiene), SPMA (~7.6 fold) (metabolite of benzene), and HEMA (~2.5 fold) (metabolite of ethylene oxide), in TTS compared to NS, supporting the relevant contribution of tobacco smoking to the body burden of these carcinogens. Among probable carcinogens (IARC Group 2A), our results show a substantial contribution of tobacco smoking to the internal dose of N,N-dimethylformamide (higher levels of AMCC), styrene (higher levels of PHEMA) and acrylamide, assessed with the levels of AAMA and GAMA (Table 4). The latter metabolites were estimated to account for 51.7% and 4.6% of the adsorbed dose (Hartmann et al., 2009) and this proportion is in agreement with our results. EMA and MMA are generic metabolites of ethylating and methylating agents, respectively, such as the probable carcinogens N-nitrosodiethylamine, and N-nitrosodimethylamine (IARC, 1978) (Table 1). Significant higher levels of EMA were found in TTS than in NS, in contrast with a previous study (Pluym et al., 2015) (Supplementary Table S2), while MMA was comparable among groups, in line with another study (Eckert and Göen, 2014), indicating that this metabolite is not able to discriminate for different smoking habits. Among possible carcinogens (IARC group 2B), CEMA, metabolite of acrylonitrile, was significantly different among all groups (□165- and □5 fold higher in TTS and ECU than NS, respectively), 2-HPMA, metabolite of propylene oxide, was higher only in TTS than NS, and NANPC, a metabolite of 4-chloronitrobenzene, was below the limit of quantitation in most samples, showing that the general population is not exposed to 4-chloronitrobenzene nor is it related to smoking habits (indeed, NANPC has been solely identified

in subjects occupationally exposed to 4-chloronitrobenzene) (Jones et al., 2007; Sabbioni et al., 2016). Finally, among toxic compounds not classifiable as carcinogens (IARC group 3), both TTS and ECU contributed to the body burden of acrolein with different intensities since 3-HPMA was about 8 fold higher in TTS than in NS and about + 80% higher in ECU than in NS. HMPMA and CMEMA, respectively major and minor metabolite of crotonaldehyde (Gray and Barnsley, 1971; Scherer et al., 2007), were significantly higher in TTS. No difference was found for SBMA, metabolite of toluene, in agreement with most studies (Alwis et al., 2012; Keith et al., 2019; Pluym et al., 2015) (Supplementary Table S2), a part from one which reported a difference between ECU and NS (Goniewicz et al., 2018). Interestingly, other biomarkers of toluene, such as toluene in blood and o-cresol in urine, were higher in TTS than in NS (Chambers et al., 2011; Fustinoni et al., 2007; Jain, 2016). The small amount of toluene biotransformed into SBMA and/or the lack of specificity may explain the result of SBMA. Indeed, p-toluylmercapturic acid, another specific mercapturic acid of toluene (Angerer et al., 1998), could be a better biomarker of tobacco smoking than SBMA.

The multiple linear regression analyses (model A) highlighted the role played by cotinine as a determinant for urinary mercapturic acids. Cotinine, a metabolite of nicotine, has a half-life of about 17 hours (Benowitz, 1996), and therefore represents exposure to nicotine over the previous days. Results revealed that cotinine is a significant determinant for most of the considered mercapturic acids (Table 4); however, it did not allow to discriminate between tobacco smoking and electronic cigarette use, as both products contain nicotine. For this reason, the linear model B was computed introducing the categorical variable “smoking mode” (NS, ECU or TTS) instead of cotinine. Overall, the adjusted coefficients of determination improved for all compounds in comparison with model A. Indeed, the variable “smoking mode” allowed us to properly take into account the presence of products of combustion processes, typical of tobacco smoking. The linear model B highlights that being a tobacco smoker is a significant determinant of exposure for the large majority of studied mercapturic acids, with an increase ranging from + 40% to 165-fold in comparison to NS. The highest increases were observed for CEMA, MHBMA, 3-HPMA, and SPMA (165, 22, 8, and 7.7-

fold, respectively) (Table 4), and among these metabolite we note the presence of those of 1,3-butadiene and benzene, both classified as known carcinogens to humans. On the other hand, ECU had higher levels of several mercapturic acids, among which 2-HPMA, 3-HPMA, AMCC, CEMA, GAMA; HEMA, MHBMA, MMA, SPMA (from +25% to 385%) (Table 4 and Fig. 1) than NS. Such increments were statistically significant for 3-HPMA and CEMA, which were about + 80% and 4.9-fold higher in ECU than in NS. These results indicate that electronic cigarette vapour is a source of exposure to acrylonitrile and acrolein, and suggest that it could be a source also of propylene oxide, N,N-dimethylformamide, ethylene oxide, 1,3-butadiene, methylating agents, and benzene, although further evidences should be provided in a study with a higher number of ECU. The ratio of the increment between TTS and ECU shows that tobacco smoking contributes to the internal dose of these chemicals from + 60% to 34-fold more than ECU. In particular, the internal dose of benzene and 1,3-butadiene in ECU, estimated as urinary mercapturic acids, accounts only for 23% and 13% of that observed in TTS.

The multiple linear regression analysis allowed us to take into consideration the role played by occupational exposure on levels of urinary mercapturic acids: it was found that plant workers had higher levels of SPMA and PHEMA, but not of other mercapturic acids, in comparison with office workers. However, SPMA levels were low and within the reference values (CDC, 2019) and comparable to previous experiences in the Italian population (Ranzi et al., 2013) and in other countries (Alwis et al., 2012; Chiang et al., 2015; Goniewicz et al., 2018; Li et al., 2015; Zhang et al., 2014). Finally, it is worth to mention that creatinine was significantly associated with an increase of all mercapturic acids included in this study, in agreement with previous findings (Eckert et al., 2011).

A few studies investigating the effect of smoking mode on the levels of urinary mercapturic acids were previously published, some of which only compared TTS and NS, while the most recent ones included also ECU. A selection of these studies is summarized in Supplementary Table S2. Generally, the number of investigated mercapturic acids was lower than that included in the present

study. Differences between TTS and NS were consistently observed in all studies for the majority of investigated mercapturic acids. ECU, if compared to NS, was associated with an increase of CEMA (Goniewicz et al., 2018; Keith et al., 2019; Rubinstein et al., 2018) and 3-HPMA, although, the latter, with fewer evidences (Keith et al., 2019; Rubinstein et al., 2018). Goniewicz and co-workers found significant differences also for AMCC and, interestingly, SBMA (Goniewicz et al., 2018), Keith and co-workers found a significant difference also in levels of AAMA (Keith et al., 2019); while, in a study conducted on adolescents, significant differences were found also for AAMA, 2-HPMA, and HMPMA (Rubinstein et al., 2018).

Comparing the concentration of mercapturic acids of the present study with those of previous studies (Supplementary Table S2), we notice some discrepancies, such as for GAMA, MHBMA, and SPMA, which were lower than those reported by other authors. This suggests critical issues associated with analytical methods applied to the determination of very low levels of mercapturic acids in urine. To tackle with the issue of accuracy, the assay used in the present work was extensively validated (Frigerio et al., 2019) and accuracy was verified by the successful participation in an external verification exercise, in which 10 urinary mercapturic acids, each tested at two different levels of occupational interest, are circulated among participating laboratories (G-EQUAS, 2019; Göen et al., 2012). However, at the moment, reference materials containing these analytes at levels of interest for the general population are lacking, while they would be useful for future applications of these biomarkers in epidemiological studies.

This study has some limitations, the major of which is the low number of study subjects, especially ECU. Although we found increases in the concentrations of several mercapturic acids in ECU compared to NS, only CEMA and 3-HPMA levels were statistically significant; it is expected that a greater sample size could highlight differences also for other mercapturic acids. Another limitation is the unequal distribution of gender, occupational exposure and smoking habits between the subgroups, which was, however, corrected through the inclusion of these variables in the linear regression models. A further issue is the lack of information about the type of electronic cigarette

used (e.g. first or more advanced generation electronic cigarettes) and about the time elapsed from the last cigarette. Among the strengths of this work there are the assessment of a large number of mercapturic acids, which can take into account for the exposure to a mixture of different toxicants and the good quality of the analytical data, as testified by the external verification exercise. Another strength of the study is the verification of several possible sources of exposure to toxicants.

In conclusion, the results of this study allowed to estimate the exposure to several toxic compounds, including some carcinogens, in subjects with different smoking habits. The biomarkers most appropriate to discriminate TTS from NS are CEMA, MHBMA, 3-HPMA, and SPMA; while those potentially able to discriminate ECU from NS and TTS are CEMA and 3-HPMA, the former, in particular, showing a very large and significant difference among groups, despite the low number of ECU involved in this study. The comparative evaluation of the contribute of different smoking modes to the internal dose of chemicals confirms that tobacco smoking is a major source of exposure to carcinogenic and non-carcinogenic chemicals, and it highlights that also electronic cigarette smoking contributes to the internal dose of several toxicants, in particular acrylonitrile and acrolein.

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Declarations of interest

None.

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Tables

Table 1

List of toxic compounds, information about their presence in tobacco smoke and electronic cigarette vapour according to the literature, and derived mercapturic acids investigated in this study.

Toxic compounds	IARC classification of carcinogenicity [a]	Presence in tobacco smoke		Presence in electronic cigarette		Urinary mercapturic acids investigated
		Mainstream smoke[b]	Sidestream smoke[b]	Vapour	Liquid	
1,3-butadiene	1	20-122.5 µg/cigarette (IARC, 2004)	81.3–250 µg/cigarette (IARC, 2004)	Not detected in the mist (Laugesen, 2008)	Detected in 2% of refill liquids (10 µg/g) (Varlet et al., 2015)	DHBMA MHBMA
4-chloronitrobenzene	2B	NF	NF	NF	NF	NANPC
acrolein	3	51.2–223.4 µg/cigarette (IARC, 2004)	342.1–1000 µg/ cigarette (IARC, 2004)	Not detected in e-cigarette mist (Laugesen, 2008) N.D. - 41.9 µg/150 puffs (Goniewicz et al., 2014) N.D. - 36 mg/m^3 (Uchiyama et al., 2013) 0.17 – 3.70 µg/cigarette equivalent (Papoušek et al., 2014) 120 - 10060 ng/mg in mainstream vapour (mass per e-liquid consumed) (Sleiman et al., 2016)	Detected in 7% of refill liquids; in those positive: 0.18 - 1.03 µg/g (Varlet et al., 2015)	3-HPMA
acrylamide	2A	Present (IARC, 2004)	NF	No detectable levels (Papoušek et al., 2014)	NF	AAMA GAMA
acrylonitrile	2B	3-39.1 µg/cigarette(IARC, 2004)	24.1–43.9 µg/cigarette (IARC, 2004)	NF	NF	CEMA HEMA
benzene	1	12–105.9 µg/cigarette (IARC, 2004)	70.7–529 µg/cigarette (IARC, 2004)	Not detected in e-cigarette mist (Laugesen, 2008) <1 µg/m^3 emission test chamber measurement (Schripp et al., 2013)	Detected in 24% of nicotine liquids; in those positive: 0.008 - 2.28 mg/L (Lim and Shin, 2017)	SPMA

				34 – 440 ng/mg in mainstream vapour (mass per e-liquid consumed) (Sleiman et al., 2016)		
crotonaldehyde	3	11.6–66.2 µg/cigarette (IARC, 2004)	62.2–121.8 µg/cigarette (IARC, 2004)	N.D - 720 ng/mg in mainstream vapour (mass per e-liquid consumed) (Sleiman et al., 2016)	Detected in 5% of refill liquids; in those positive: 0.067 - 0.084 µg/g) (Varlet et al., 2015)	CMEMA HMPMA
N-nitrosodiethylamine and others ethylating agents	2A	ND–25 ng (IARC, 2004)	NF	NF	NF	EMA
ethylene oxide	1	7 µg/cigarette (IARC, 2004)	NF	NF	Detected in 5% of refill liquids; in those positive: 9 - 13 µg/g) (Varlet et al., 2015)	HEMA EMA
N-nitrosodimethylamine and others methylating agents	2A	0.1–180 ng/cigarette (IARC, 2004)	NF	NF	NF	MMA
N,N-dimethylformamide	2A	Present in tobacco smoke (Talhout et al., 2011)	NF	NF	NF	AMCC
propylene oxide	2B	0–100 ng/cigarette (IARC, 2004)	NF	Not detected in e-cigarette mist (Laugesen, 2008)	4.2 - 6.7 mg/mL in E-Liquid (Sleiman et al., 2016)	2-HPMA
styrene	2A	4.5–19.3 µg/cigarette (IARC, 2004)	23.2–46.1 µg/cigarette (IARC, 2004)	0.29 ppm in 38 mL sample of e-cigarette mist (Laugesen, 2008)	Detected in 11% of flavoured and nicotine liquids; in those positive: 0.011 - 0.201 mg/L (Lim and Shin, 2017)	PHEMA
toluene	3	48.3–173.7 µg/cigarette (IARC, 2004)	134.9–1060 µg/cigarette (IARC, 2004)	< 1 µg/m^3 emission test chamber measurement (Schripp et al., 2013) ND - 6.3 µg/150 puffs (Goniewicz et al., 2014)	Detected in 17 % of flavoured and nicotine liquids; in those positive: 0.006 - 0.687 mg/L (Lim and Shin, 2017)	SBMA

NF: information not found in the literature

ND: not detected

a IARC classification: Group 1, carcinogenic to humans; Group 2A, probably carcinogenic to humans; Group 2B, possibly carcinogenic to humans; Group 3; not classifiable as to its carcinogenicity to humans

b Mainstream smoke is the smoke released at the mouth end of the cigarette during puffing while sidestream smoke is the smoke released from the burning cone and through the cigarette paper (IARC, 2004).

Table 2

Main personal characteristics and information on smoking habits of the study subjects grouped into non-smokers (NS), electronic cigarette users (ECU), and tobacco smokers (TTS, according to cotinine cut off ≥30 µg/L).

		NS	**ECU**	**TTS**
Smoking status*	N	38	7	22
Intensity of tobacco smoking* (cigarettes/day)	Mean (min-max)	-	-	13 (2 - 25)
nicotine (µg/L)	Median (5^{th} - 95^{th}) %>LOQ	0.11 (<0.10 - 1.63) 50	2003 (537 - 4486) 100	1456 (225 - 5120) 100
cotinine (µg/L)	Median (5^{th} - 95^{th}) %>LOQ	0.35 (<0.10 - 1.93) 71	1530 (1179 - 2772) 100	1772 (601 - 4000) 100
creatinine (g/L)	Median (5^{th} - 95^{th})	1.5 (0.4 - 2.6)	1.5 (0.5 - 2.5)	1.4 (0.6 - 2.8)
Male gender	N	34	7	22
Plant workers	N	32	7	19
Plant workers and male gender	N	30	7	19
Age (y)	Mean (min-max)	46 (28 - 62)	46 (37 - 55)	45 (27 - 57)
BMI (kg/m^2)	Mean (min-max)	26.2 (19.6 - 37.0)	25.9 (22.8 - 30.8)	26.2 (19.0 - 30.5)

* after correction according to cotinine level evaluation

Table 3

Concentration of mercapturic acids in urine samples (µg/g creatinine), expressed as median, 5th and 95th percentile, and % of samples >LOQ, in subjects grouped by smoking habit, together with results of Anova test and Bonferroni *post-hoc* test.

Urinary mercapturic acids	**LOQ (µg/L)**	**statistics**	**NS (µg/g creatinine)**	**ECU (µg/g creatinine)**	**TTS (µg/g creatinine)**	***p*-Value (Anova)**	***p*-Value NS vs ECU**	***p*-Value NS vs TTS**	***p*-Value ECU vs TTS**
2-HPMA	0.5	Median	8.8	9.8	28.4	< 0.001	1.000	< 0.001	< 0.001
		5th - 95th	4.2 - 16.4	6.7 - 17.4	9.4 - 70.9				
		%>LOQ	100	100	100				
3-HPMA	0.2	Median	160.6	222.1	1301.2	< 0.001	0.069	< 0.001	< 0.001
		5th - 95th	77.9 - 318.5	196.6 - 738.2	328.9 - 3661.1				
		%>LOQ	100	100	100				
AAMA	3.2	Median	47.9	55.8	114.6	< 0.001	1.000	< 0.001	< 0.001
		5th - 95th	24.2 - 95.4	34.4 - 65.5	55.1 - 223.9				
		%>LOQ	100	100	100				
AMCC	2	Median	142	243	405	< 0.001	1.000	< 0.001	0.105
		5th - 95th	55 - 434	60 - 519	90 - 844				
		%>LOQ	100	100	100				
CEMA	0.9	Median	0.9	2.7	163.1	< 0.001	<0.001	< 0.001	< 0.001
		5th - 95th	<LOQ - 2.1	0.9 - 36.5	45.8 - 358.4				
		%>LOQ	63	86	100				
CMEMA	2	Median	273	233	400	0.018	1.000	0.031	0.100
		5th - 95th	122 - 603	154 - 542	220 - 774				
		%>LOQ	100	100	100				

DHBMA	1.0	Median	247.5	263.8	479.1	< 0.001	1.000	< 0.001	< 0.001
		5th - 95th	163.6 - 348.5	177.3 - 298.7	273.2 - 925.6				
		%>LOQ	100	100	100				
EMA	0.01	Median	0.03	0.03	0.06	0.033	1.000	0.029	0.615
		5th - 95th	<LOQ - 0.11	<LOQ - 0.10	<LOQ - 0.80				
		%>LOQ	55	57	64				
GAMA	1.0	Median	2.5	3.9	5.3	0.002	0.974	0.001	0.501
		5th - 95th	<LOQ - 7.1	1.4 - 6.7	1.7 - 30.4				
		%>LOQ	84	100	95				
HEMA	0.3	Median	1.3	2.0	3.2	0.002	0.872	0.002	0.657
		5th - 95th	0.1 - 4.1	1.3 - 2.2	1.0 - 26.7				
		%>LOQ	95	100	100				
HMPMA	2	Median	48	38	268	< 0.001	1.000	< 0.001	< 0.001
		5th - 95th	15 - 265	19 - 133	96 - 580				
		%>LOQ	100	100	100				
MHBMA#	0.04	Median	0.27	0.55	4.07	< 0.001	0.410	< 0.001	0.016
		5th - 95th	<LOQ - 2.47	0.14 - 2.07	0.74 - 11.38				
		%>LOQ	63	100	100				
MMA	0.09	Median	2.57	4.70	2.64	0.662	1.000	1.000	1.000
		5th - 95th	0.36 - 10.52	1.64 - 7.15	0.70 - 17.39				
		%>LOQ	97	100	100				
NANPC	0.11	Median	<LOQ	<LOQ	<LOQ	N.A	N.A	N.A	N.A
		5th - 95th	<LOQ - 0.16	<LOQ - 0.11	<LOQ - <LOQ				
		%>LOQ	13	14	5				

PHEMA[#]	0.01	Median	0.53	0.68	1.05	0.003	1.000	0.002	0.138
		5th - 95th	0.09 - 1.36	0.17 - 1.29	0.39 - 2.55				
		%>LOQ	100	86	100				
SBMA	0.02	Median	2.22	1.42	1.47	0.096	0.472	0.158	1.000
		5th - 95th	0.55 - 12.74	0.40 - 4.28	0.53 - 2.96				
		%>LOQ	100	100	100				
SPMA	0.01	Median	0.06	0.16	0.48	< 0.001	0.560	< 0.001	0.001
		5th - 95th	<LOQ - 0.23	0.03 - 0.34	0.08 - 1.45				
		%>LOQ	92	86	100				

N.A. Not assessed

#As a sum of isomers

Table 4

Results of multiple linear regression models A and B in which the concentration of each urinary mercapturic acid is the dependent variable. In the linear model A the independent variables are: cotinine (log_{10} transformed, µg/L), creatinine (log_{10} transformed, g/L), age (years), gender (male = reference, female), BMI (kg/m^2), and occupational exposure (office workers = reference, plant workers). In the linear model B urinary cotinine was replaced by smoking mode (NS = reference, ECU, TTS). Results are reported as Geometric Mean Ratio (GMR), with respective 95% confidence interval (95% CI), obtained performing exponentiation on the relatives adjusted slopes.

Urinary mercapturic acids	**Linear model A**			**Linear model B**		
	Cotinine			ECU	TTS	
	GMR (95% CI) *p*-Value	r (95% CI) *p*-Value	R^2_{adj} *p*-Value	GMR (95% CI) *p*-Value	GMR (95% CI) *p*-Value	R^2_{adj} *p*-Value
2-HPMA	1.30 1.20 - 1.42 <0.001	0.62 0.45 - 0.75 <0.001	0.55 <0.001	1.30 0.80 - 2.13 0.289	3.56 2.57 - 4.91 <0.001	0.63 <0.001
3-HPMA	1.54 1.39 - 1.70 <0.001	0.75 0.62 - 0.84 <0.001	0.68 <0.001	1.78 1.09 - 2.90 0.021	8.14 5.91 - 11.22 <0.001	0.81 <0.001
AAMA	1.20 1.13 - 1.28 <0.001	0.60 0.41 - 0.73 <0.001	0.66 <0.001	1.07 0.74 - 1.53 0.720	2.42 1.91 - 3.07 <0.001	0.73 <0.001
AMCC	1.23 1.12 - 1.35 <0.001	0.50 0.30 - 0.66 <0.001	0.61 <0.001	1.25 0.70 - 2.24 0.437	2.62 1.79 - 3.84 <0.001	0.63 <0.001
CEMA	2.92 2.46 - 3.47 <0.001	0.85 0.76 - 0.90 <0.001	0.72 <0.001	4.85 2.68 - 8.77 <0.001	164.97 111.66 - 243.73 <0.001	0.92 <0.001
CMEMA	1.07 1.00 - 1.14 0.051	0.25 0.01 - 0.46 0.042	0.63 <0.001	0.90 0.59 - 1.36 0.605	1.44 1.09 - 1.89 0.010	0.65 <0.001
DHBMA	1.13 1.07 - 1.20 <0.001	0.50 0.29 - 0.66 <0.001	0.67 <0.001	0.93 0.69 - 1.27 0.662	2.02 1.65 - 2.48 <0.001	0.77 <0.001
EMA	1.20 1.01 - 1.42	0.27 0.03 - 0.48	0.11 0.044	1.10 0.37 - 3.23	2.51 1.23 - 5.10	0.13 0.035

	0.035	0.028		0.866	0.012	
GAMA	1.19 1.08 - 1.32 0.001	0.40 0.18 - 0.59 0.001	0.43 <0.001	1.42 0.73 - 2.77 0.291	2.08 1.34 - 3.22 0.001	0.42 <0.001
HEMA	1.27 1.12 - 1.43 <0.001	0.45 0.23 - 0.62 <0.001	0.18 0.005	1.72 0.79 - 3.75 0.171	2.78 1.66 - 4.65 <0.001	0.18 0.007
HMPMA	1.36 1.21 - 1.53 <0.001	0.55 0.36 - 0.70 <0.001	0.48 <0.001	0.88 0.47 - 1.65 0.691	5.26 3.49 - 7.93 <0.001	0.66 <0.001
MHBMA	1.85 1.51 - 2.28 <0.001	0.61 0.43 - 0.74 <0.001	0.46 <0.001	2.56 0.79 – 8.28 0.114	21.99 10.16 - 47.61 <0.001	0.58 <0.001
MMA	1.08 0.95 - 1.23 0.238	0.15 -0.09 - 0.38 0.220	0.39 <0.001	1.72 0.75 - 3.96 0.198	1.24 0.72 - 2.15 0.435	0.38 <0.001
PHEMA	1.23 1.09 - 1.39 <0.001	0.41 0.19 - 0.59 <0.001	0.33 <0.001	1.00 0.46 - 2.15 0.997	2.56 1.54 - 4.23 <0.001	0.35 <0.001
SBMA	0.90 0.81 - 1.01 0.073	-0.23 -0.44 - 0.01 0.062	0.32 <0.001	0.61 0.30 - 1.27 0.183	0.65 0.40 - 1.05 0.076	0.32 <0.001
SPMA	1.53 1.34 - 1.75 <0.001	0.64 0.47 - 0.76 <0.001	0.48 <0.001	1.51 0.70 - 3.25 0.285	7.65 4.62 - 12.66 <0.001	0.58 <0.001

Figure legends

Fig 1-A and **1-B.** Boxplots representing the distribution of fitted values obtained from the multiple linear model B for all investigated mercapturic acids. Data reported are corrected for creatinine, age, gender, BMI, and occupational exposure. The box contains the 50% of the observations, with the median dividing the box in two areas. The upper and lower hinge represent the 25th and 75th percentile of the distribution, respectively. Outside the box, the upper whisker extends from the hinge to the highest value no further than 1.5 * interquartile range (IQR) from the hinge. The lower whisker extends from the hinge to the smallest value at most 1.5 * IQR of the hinge. Data beyond the whiskers are plotted individually as dots.